Evidence for Nucleosomal Phasing and a Novel Protein Specifically Binding to Cucumber Satellite DNA

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The nucleosomal organization and the protein-binding capability of highly repeated and methylated satellite DNA of cucumber (*Cucumis sativus* L.), comprising approx. 30% of the genome, were analyzed. Nucleosomal core DNA from satellite type I was prepared after micrococcal nuclease digestion of chromatin and sequenced. Most of the core sequences obtained could be grouped in two main (A and B) and two minor groups (C and D) indicating a specific and complex phasing of nucleosomes on this satellite DNA. *In vitro,* gel retardation assays with cloned satellite DNA repeats (types I–IV) demonstrated a specific binding of nuclear proteins. These specific binding effects are also obtained with genomic, *in vivo* methylated and sequence heterogeneous (1 to 10% diversity) satellite type I DNA. For the first time in plants, a satellite DNA-binding protein with an apparent molecular weight of 14 kDa (SAT 14) was identified.

Introduction

Tandem-arranged highly repeated satellite DNA is a common component of eukaryotic genomes [1]. Higher plants are often characterized by a very large amount of satellite DNA [2] packaged in condensed heterochromatin. However, the process of chromatin packaging leading to condensed heterochromatin is still unknown. The lengths of satellite DNA repeats in plants (and in many animals) mainly vary between 160 and 185 base pairs (bp) or 350 and 360 bp, respectively (compiled in [3]). This is in the range of the length of a nucleosomal DNA repeat or a nucleosome dimer: core DNA folded around the histone octamer plus linker DNA in plants is determined to be 175 to 185 bp in length [4, 5]. The coincidence in lengths led to the assumption that nucleosomes might show a characteristic and specific arrangement ("phasing") in plant satellite chromatin.

Van Holde distinguished spacing, positioning and phasing of nucleosomes along the DNA strand [6]. Here positioning means that the site of the nucleosome is fixed either by DNA-histone interaction or by sequence-specific DNA-binding proteins (for a review see [7]). For tandem-arranged repeated DNA, positioning of nucleosomes results in phasing as already shown for the 172-bp alpha-satellite DNA of African green monkey [8–11], for the AT-rich satellite DNA of mouse [12, 13] or the satellite I of rat [14, 15]. In the case of African green monkey alpha-satellite DNA, a specifically binding protein was characterized [16] which was shown later to interact with any stretch of six A–T base pairs in duplex DNA [17]. The first satellite DNA-binding proteins described were an unpurified protein [18] and later the D1 protein of *Drosophila melanogaster* [19, 20]. Furthermore, minisatellite DNA-binding proteins Msbp 1–3 of mouse and other eukaryotes [21, 22], an AT-binding protein of *Dictyostelium discoideum* [23], and the MAP2 protein of mouse [24] were characterized. For none of them a function has actually been proved. Recently, a 84 kDa protein has been purified (MeCP2) from nuclear extracts of rat cells which binds specifically to methylated DNA [25].

Plant satellite DNA, often highly methylated and packaged as condensed heterochromatin, has not been investigated yet with respect to specifically binding proteins. Only one plant protein has been identified in pea nuclear extracts which recognizes 5-methylcytosine residues in DNA without appreciable sequence specificity [26]. Therefore, we investigated the distribution of nucleosomes on plant satellite DNA which may be influenced by specific nuclear non-histone proteins.

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Nuclear genomes of representatives of the plant family Cucurbitaceae often contain large amounts of highly repeated satellite DNA [27]. In cucumber (*Cucumis sativus* L.) highly methylated satellite DNA repeats comprise approx. 30% of the nuclear genome [28] probably organized as heterochromatic blocks in the interphase nuclei and as heterochromatic bands within the metaphase chromosomes [29]. Four related satellite DNA types were described: type I/II (182 bp), type III (177 bp) and type IV (360 bp) [30, 31]. Types I and II are only discriminated by a specific Taq I site. Types I/II and III show homologies of approx. 60% among each other; type IV consists of a stretch of 180 bp with 60–65% sequence similarity to type I as well as to type III and of a non-homologous part of 180 bp (Fig. 1). Nucleosomal core DNA of satellite type I obtained after *Micrococcus* nuclease/exonuclease III digestion of cucumber chromatin and subsequent treatment with mung bean nuclease was cloned and sequenced. A complex, but non-random arrangement of nucleosomes could be deduced from the aligned sequences. Furthermore, evidence for the existence of at least one specifically satellite DNA-binding protein with an apparent molecular weight of 14 kDa (sat 14) was obtained.

Methods

Plant material

Seeds of cucumber (*Cucumis sativus* L. *cv.* Sensation Typ Neckarruhm) were purchased from Fa. Endriß, Tübingen, F.R.G. Seeds were washed with ethanol for 30 min, subsequently treated with 1% sodium hypochlorite for 30 min, washed several times with sterile dest. H_2O, and grown in daylight at 25 °C for 7 days under sterile conditions. The cotyledons were harvested, frozen with liquid nitrogen and stored at –70 °C.

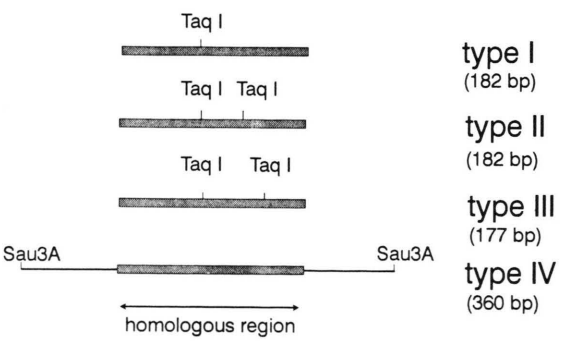

Fig. 1. Organization and comparison of the four related repeat types of cucumber satellite DNA. Type I/II and type III each exhibit approximately 65% homology to the 180 bp region of type IV [30, 31]. The respective satellite DNA repeat characterizing restriction enzyme sites are indicated.

Isolation of satellite DNA

Nuclei were isolated from seedlings and DNA was purified by a combination of CsCl and CsCl-actinomycin D buoyant density gradients using the method described by Hemleben *et al.* [28]. Corresponding satellite DNA fractions were precipitated with isopropanol, and Taq I-digested DNA was separated on 8% polyacrylamide gels [30]. The 182-bp satellite type I repeat was eluted out of the gel, gamma-^{32}P-end-labelled and applied in gel retardation assays.

For cloned satellite repeats of type I, the insert of clone pSG 12 (182-bp type I insert) was used [30]. Satellite type III (177 bp and type IV (360 bp) repeats described by Ganal *et al.* [30] and Ganal and Hemleben [31] were obtained using the inserts of the clones pSHS 2/5 (type III) and pSHS 1/76 (type IV).

Type I satellite DNA used for coupling to CNBr-activated sepharose 4 B was amplified by PCR, following standard protocols.

Preparation of nucleosomal core DNA and cloning

Frozen cotyledons (5 g) were homogenized in liquid nitrogen. Nuclei were isolated from the resultant powder as described by Hemleben *et al.* [28] and suspended in 1.4 ml *Micrococcus* nuclease buffer (100 mM NaCl, 66 mM $MgCl_2$, 1 mM $CaCl_2$, 25 mM sucrose, 10 mM Tris-HCl, pH 7.5). 200 µl aliquots were incubated with 40 units *Micrococcus* nuclease for 15 min at 37 °C. Aliquots were checked for approx. 180-bp bands by Southern blotting and hybridization with radioactively labelled satellite type I DNA of cucumber. Subsequently, the linker DNA was hydrolyzed to single-stranded DNA with exonuclease III until the nucleosomal core was reached; this procedure was checked by agarose gel electrophoresis. Aliquots showing an obvious stop of exonuclease III action were used for cloning after deproteinization and removal of overhanging single-stranded DNA by mung bean nuclease. Therefore, after adding 100 units exonuclease III

the aliquots were incubated for 15 min at 37 °C, then extracted with phenol and phenol/chloroform and the DNA was precipitated, solved in 200 µl mung bean nuclease buffer (0.3 M sodium acetate, pH 4.6, 0.5 M NaCl, 10 mM $ZnCl_2$), and incubated with 20 units mung bean nuclease for 20 min at 37 °C. Cloning of the blunt-end core DNA fragments was carried out into Sma I- or Hinc III-digested pUC 18/19 vectors [32], and satellite type I clones were identified by colony filter hybridization.

DNA sequencing

For nucleotide sequencing of 35 independent clones of core satellite type I DNA the method of Sanger *et al.* [33] was used. Computer analysis of the sequences was carried out by application of the program IBI "DNA/protein sequence analysis" [34].

Preparation of nuclear protein extracts

Frozen cotyledons (120 g) were homogenized in liquid nitrogen. Nuclei were isolated from the resultant powder as described by Hemleben *et al.* [28]. The nuclei were washed twice and then stirred for 30 min at 4 °C in 5 ml of extraction buffer (buffer D: 20 mM HEPES, pH 8.0, 40 mM KCl, 5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol) containing 0.42 M NaCl. This crude nuclear extract was clarified by centrifugation (12,000 rpm, 20 min, SS 34 rotor, SORVALL); the supernatant was ultrafiltrated using Centricon tubes (centrifugation at 7500 rpm) until the final protein concentration of about 30 µg/µl [35] was reached. The protein extract was extensively dialyzed against buffer D containing 20% glycerol, and stored at −20 °C.

Gel retardation assay

Aliquots of crude nuclear proteins were preincubated each with an equal amount of sonicated calf thymus DNA in DNA-binding buffer (buffer B: 50 mM NaCl, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM dithioerythrol, 5% glycerol) for 15 min at room temperature. 5 ng of ^{32}P-end-labelled DNA fragments (and 200-fold excess of unlabelled competitor DNA as indicated) were added, and the mixture was then incubated for further 15 min. The DNA-protein complexes were separated on 5% polyacrylamide gels (acrylamide : bisacrylamide, 47 : 1) overnight at 40 V, and the dried gels subsequently exposed to X-ray films. PCR products were end-labelled with gamma-^{32}P-dATP and T4 kinase, other DNA fragments were end-labelled with alpha-^{32}P-dATP and alpha-^{32}P-dCTP using Klenow polymerase.

Characterization of protein components of DNA-protein complexes

The DNA-protein complexes detected in the gel retardation assays were cut out of the dried gels, and the proteins were analyzed as described by Zentgraf and Hemleben [36].

Affinity column chromatography

100 µg of PCR-amplified satellite type I DNA fragments were coupled to 1.5 g CNBr-activated sepharose 4 B (Pharmacia) in accordance with manufacturer instructions. The sepharose column was equilibrated with DNA-protein-binding buffer B, loaded with 5 mg crude nuclear protein extract, which was preincubated with 5 mg sonicated calf thymus DNA, and incubated for 1 h at room temperature. Then the column was washed with 100 ml buffer B; the DNA-binding proteins were eluted with buffer D containing 0.5 M KCl. (The column equilibrated with buffer D containing 0.02% sodium azide was storable at 4 °C for several month.) The protein fraction eluted from the column was analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

Results

Nucleosomal phasing on cucumber satellite DNA type I

Micrococcus nuclease treatment of cucumber chromatin, DNA purification and hybridization of Southern blotted DNA with satellite type I/II DNA (182 bp in length) resulted in nucleosomal DNA ranging from monomers to multimers of a basic repeat length of approx. 180 bp demonstrating that satellite DNA is packaged in a nucleosomal structure (data not shown). In order to investigate whether a defined region of the satellite DNA repeat is protected by the histone octamer, cucumber nuclei were treated with *Micrococcus* nuclease/exo-

nuclease III to obtain nucleosomal core DNA fragments. This procedure was chosen to prevent the development of artefacts resulting from sequence specifity of *Micrococcus* nuclease [13]. Double-strand cuts introduced by *Micrococcus* nuclease into the linker region were used as starting points for exonuclease III hydrolysis resulting in overhanging 5′ ends of single-stranded DNA. After deproteinization the single-strands were removed by mung bean nuclease digestion. Core DNA (mean length approx. 145 bp) was size-fractionated by agarose gel electrophoresis, blunt-end cloned, and satellite type I/II clones were identified by colony filter hybridization. The inserts, mostly 142 to 146 bp in length, of 35 independent clones were sequenced, and the sequences were aligned with two adjacent satellite DNA type I repeats (numbered from nts 1 to 364).

Interestingly, 30 of the core DNA sequences could be subdivided into two major and two minor characteristic groups (Fig. 2 A): preferentially the region of nts 160 to 302 (group A) and the region of nts 136 to 279 (group B) were protected by nucleosomes; in the two minor groups core DNA ranged from nts 63 to 209 (group C) or from nts 166 to 309 (group D). Obviously, different but defined phasing frames of the 182-bp satellite type I/II repeats were observed indicating a differential and complex phasing pattern of nucleosomes within cucumber satellite chromatin. Five of the core satellite DNAs sequenced did not fit into either of these phasing frames.

Comparing the sequences of type I/II core DNA the extent of sequence heterogeneity among the repeats generally was 1–10%. However, base exchange sites are obviously non-randomly distributed; certain regions within the repeats appeared to be more conserved than others (Fig. 2 C). The five core satellite DNAs exhibiting different phases showed a higher sequence heterogeneity, *i.e.* up to 19% deviation from the reference sequence. In Fig. 2 D those regions of the satellite types I/II, III and IV are indicated which exhibits higher sequence similarity among each other (according to [31]).

Binding of nuclear proteins to satellite DNA

With this evidence for a preferential distribution of nucleosomes (phasing) in cucumber satellite chromatin the question arose whether – in addition to histones – nuclear proteins might exist which interact specifically with satellite DNA. Such proteins could be involved in the observed phasing effects *in vivo*.

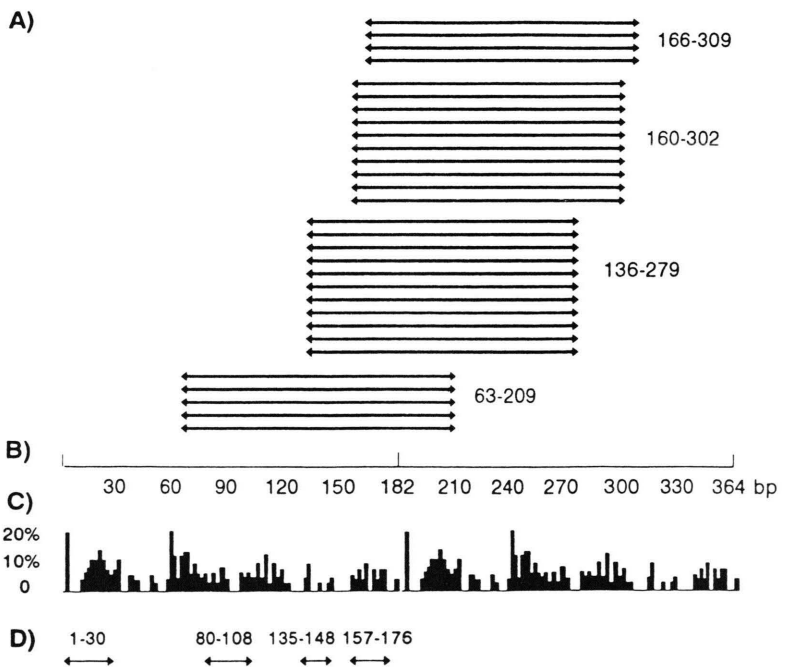

Fig. 2. Nucleosomal phasing and sequence variability of satellite DNA of cucumber. Nucleosomal core DNA of satellite DNA type I/II prepared by a combined treatment with micrococcal nuclease, exonuclease III and mung bean nuclease was cloned and sequenced. A: alignment of 30 nucleosomal core DNA sequences; B: two adjacent head-to-tail repeats of the reference sequence of satellite type I; C: sequence variability (%) of 35 independent cloned core DNAs shown as the number of base exchanges compared to the 182-bp satellite type I reference sequence [30]; D: conserved regions of the related cucumber satellite types III and IV compared to satellite I (according to [31]).

Nuclei of cucumber were extracted with 0.42 M salt, a concentration used to avoid extensive elution of histones from chromatin. These protein preparations were applied for gel retardation assays with a cloned satellite DNA repeat type I (Fig. 3). Increasing amounts of nuclear proteins, up to 50 μg, resulted in higher quantities of a DNA-protein complex (lanes 1–5). Specific competition with unlabelled satellite DNA type I displaced the labelled DNA of the DNA-protein complex into the unbound form (lane 6). An 141-bp restriction fragment of pUC19 used as unspecific control DNA was not able to form any DNA-protein complex with cucumber nuclear proteins (lane 10).

To test the validity of this assay and to distinguish between different binding specificities, competitions with a 200-bp ribosomal DNA promoter fragment of cucumber, shown to interact specifically with other nuclear proteins [36], and with a 123-bp calf satellite DNA were carried out. No competition effect was produced by the addition of a 200-fold excess of the respective DNA (Fig. 3; lanes 7 and 8). Therefore, evidence was obtained for the occurrence of nuclear proteins that specifically bind to satellite DNA type I.

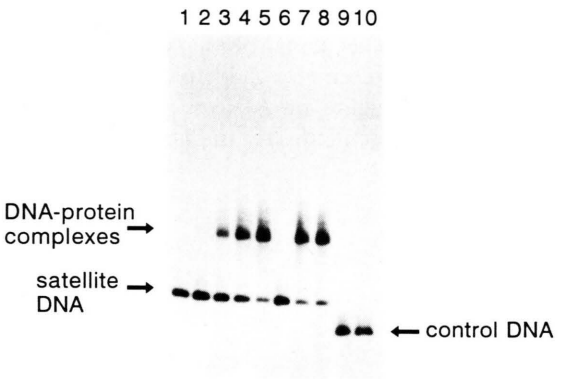

Fig. 3. Gel retardation assay with cloned and ^{32}P-end-labelled cucumber satellite DNA type I. 0.42 M salt nuclear protein extract was tested for its ability to bind specifically to satellite DNA type I. Lanes 1–5, addition of increasing amounts of nuclear proteins; 1: without nuclear extract, 2: 10 μg, 3: 15 μg, 4: 30 μg, 5: 50 μg; lanes 6–8: 50 μg nuclear protein and competition with 200-fold excess of unlabelled satellite DNA type I (lane 6), 200-bp promoter fragment of cucumber ribosomal DNA (lane 7), and 123-bp calf satellite DNA (lane 8). An end-labelled 141-bp fragment of pUC19 was incubated as unspecific control without nuclear proteins (lane 9), and with 50 μg nuclear proteins (lane 10).

Furthermore, the capability of type III and type IV satellite DNA to form a DNA-protein complex with nuclear proteins was tested in another series of gel retardation assays. Actually, all satellite types showed specific protein binding proved by competition with an excess of the respective unlabelled repeat type (Fig. 4).

Protein binding to in vivo methylated satellite DNA type I

Satellite DNA of cucumber is highly methylated at cytosine residues, and it exhibits a sequence variability among the type I repeats of approx. 1–10% ([28, 30]; Fig. 2). The potential influence of these factors upon the observed protein-binding effects has been investigated. For this purpose, genomic satellite DNA was enriched by CsCl-actinomycin D buoyant density gradients; the 182-bp Taq I digested type I satellite DNA was eluted from 8% PAGE, radioactively labelled and used for gel retardation assays (Fig. 5). This DNA clearly bound nuclear proteins (lane 2); an excess of unlabelled, cloned (unmethylated) satellite DNA type I (lane 3) could displace the labelled *in vivo* methylated DNA in the DNA-protein complex. This effect was also observed with an excess of type III (lane 4) and, slightly less, with the 360 bp type IV satellite DNA (lane 5). Apparently, the same proteins are responsible for the binding effects observed with the different satellite DNA types. At least *in vitro,* the DNA-protein complex formation appeared to be independent of cytosine methylation and sequence variability.

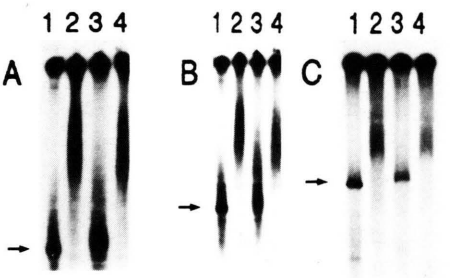

Fig. 4. Gel retardation assays with the different cucumber satellite DNA types I (A), III (B) and IV (C). Lane 1: without nuclear proteins; lane 2: 64 μg nuclear protein added; lane 3: 64 μg nuclear protein and 200-fold excess of unlabelled satellite DNA of the respective type as specific competitor; lane 4: 64 μg nuclear protein with 200-fold excess of unspecific, sonicated calf thymus competitor DNA added. The arrows point to the unbound DNA.

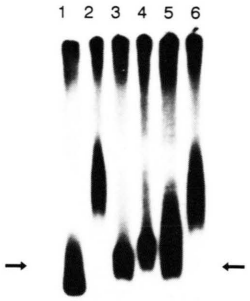

Fig. 5. Gel retardation assays with *in vivo* methylated genomic satellite DNA type I and competition with the different cloned (unmethylated) satellite DNA types I, III and IV. In all assays the ^{32}P-labelled genomic Taq I satellite DNA fragment was applied. Lane 1: without nuclear proteins; lanes 2–6: 64 µg nuclear proteins added; specific competition with 200-fold excess of unlabelled satellite DNA type I (lane 3), type III (lane 4) or type IV (lane 5); lane 6: addition of 200-fold excess of unlabelled, unspecific calf thymus DNA as competitor. The arrows point to the unbound DNA.

Characterization of satellite DNA-binding proteins

For identification of the proteins showing this specific binding capability, a 0.42 M salt nuclear extract was applied to CNBr-activated sepharose 4 B affinity chromatography columns with PCR-amplified satellite DNA type I as ligand. Protein bound by type I DNA was eluted with 0.5 M KCl. A prominent protein with an apparent molecular weight of 14 kDa (and in some cases minor bands of 13.5, 18 and 39 kDa) was detected after SDS-PAGE (Fig. 6, lane 2). This protein (SAT 14) was able to bind satellite DNA type I in a gel retardation assay (not shown).

In a different approach the protein component of the DNA-protein complex detected by gel retardation assays with satellite type I DNA was subjected to SDS-PAGE (Fig. 7). Again the 14 kDa protein appeared prominently.

Discussion

Distribution of nucleosomes on satellite DNA type I/II was shown by nucleotide sequencing of satellite core DNA obtained after micrococcal nuclease digestion of cucumber chromatin. *Micrococcus* nuclease is commonly used for such experiments but known to exhibit sequence specifities [37, 38] that might lead to artefacts with respect to nucleosomal phasing. To prevent such artefacts we used a moderate *Micrococcus* nuclease/exonuclease III treatment with subsequent mung bean nuclease digestion (according to [13]) supplying sufficient mononucleosomes but leaving the core DNA untouched. After cloning satellite core DNA type I/II inserts mainly 142 to 146 bp long (mean length: 145 bp) were obtained similar to animal nucleosomal core DNA which were shown to be 145 to 146 bp in length [6]. The fact that two major and two minor specific nucleosomal phases were observed to be prominent resembles the situation described for the alpha-satellite of African green monkey where eight different, strictly defined nucleosome

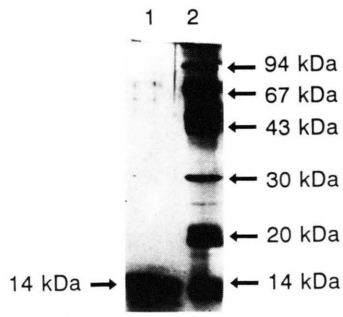

Fig. 6. SDS-PAGE of the 0.5 M KCl eluate of a CNBr-activated sepharose 4 B affinity column coupled with satellite DNA type I. Protein fractions were separated on 15% SDS-PAGE and silver-stained. Mainly a 14 kDa protein was eluted from the column. Lane 1: concentrated 0.5 M KCl eluate; lane 2: low-molecular weight protein size marker.

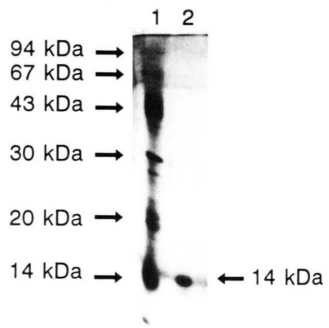

Fig. 7. SDS-PAGE of the protein component of a satellite type I DNA-protein complex from gel retardation assays. The 14 kDa protein (sat 14) is the prominent protein component binding to satellite DNA. Lane 1: low-molecular weight protein size marker; lane 2: protein eluted from the satellite DNA-protein complex detected after gel retardation assays (see Fig. 3).

positions were detected [11]. Possibly, different clusters of satellite DNA on the chromosomes show a variable nucleosomal phasing pattern.

In vitro, gel retardation experiments carried out with the cucumber satellite DNA types I, III and IV and crude nuclear protein extracts showed specific DNA-protein binding with all repeat types. Nucleotide sequence comparison between the different satellite repeats detected regions of higher similarity alternating with more non-conserved regions as indicated in Fig. 2 C and D [31]. This suggests that the rather conserved regions could represent protein-binding sites. A characteristic feature of a prominent motif present in all repeat types, 5′-CGAAAA(A)-3′, is the AT-richness assumed to play a role in DNA bending and protein binding [17, 19, 20, 39, 40]. Interestingly, specific curvature or bending can be determined in all cucumber satellite types (T. Hankeln, M. Ganal, and V. Hemleben, unpublished results). In contrast to the recently described protein MeCP2 which interacts preferentially with methylated DNA of rat and mouse [25], the specific DNA-protein interaction found for cucumber satellite DNA does occur with methylated and unmethylated (cloned) satellite type I DNA (see Fig. 5).

Characterization of the complex-forming proteins clearly demonstrated the existence of a protein with an apparent molecular weight of 14 kDa (SAT 14) consisting of one type of subunit (and perhaps some other minor proteins). It is not yet clear whether the protein binds to satellite DNA in a monomeric or multimeric form. The size of sat 14 is in the range of HMG-like proteins [6, 41]; however, those proteins are mostly involved in transcriptional activation, whereas for cucumber satellite DNA only a few transcription products could be detected (our unpublished results). Possibly, sat 14 could have a sequence-dependent positioning function for the arrangement of the following nucleosomes. A vast body of examples for positioned nucleosomes in animal cells is known [7], most of them concerning non-repetitive sequences. The above results provide the first evidence for nucleosomal phasing on plant satellite DNA; at least one specific satellite DNA-binding protein was detected. The functional correlation is subject of further investigation.

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[1] T. Beridze, Satellite DNA, Springer, Berlin, Heidelberg, New York 1986.
[2] J. Ingle, G. G. Pearson, and J. Sinclair, Nature New Biol. **242,** 193–197 (1973).
[3] V. Hemleben, U. Zentgraf, K. King, N. Borisjuk, and G. Schweizer, in: DNA-Polymorphisms in Eukaryotic Genomes (G. Kahl, H. Appelhans, J. Kömpf, and A. J. Driesel, eds.), pp. 157–170, BFT 10, Advances in Mol. Gen., Vol. 5, Hüthig Verlag, Heidelberg 1992.
[4] C. Gigot, G. Phillips, A. Nicolaieff, and L. Hirth, Nucleic Acids Res. **3,** 2315–2329 (1976).
[5] B. Leber and V. Hemleben, Nucleic Acids Res. **7,** 1263–1281 (1979).
[6] K. E. Van Holde, Chromatin, Springer, Berlin, Heidelberg, New York (1989).
[7] F. Thoma, Biochim. Biophys. Acta **11,** 1–19 (1992).
[8] P. Musich, J. J. Maio, and F. L. Brown, J. Mol. Biol. **117,** 657–677 (1977).
[9] F. Fittler and H. G. Zachau, Nucleic Acids Res. **7,** 1–13 (1979).
[10] W. Hörz, F. Fittler, and H. G. Zachau, Nucleic Acids Res. **11,** 4275–4285 (1983).
[11] X.-Y. Zhang, F. Fittler, and W. Hörz, Nucleic Acids Res. **11,** 4287–4306 (1983).
[12] X.-Y. Zhang and W. Hörz, J. Mol. Biol. **176,** 105–129 (1984).
[13] W. Linxweiler and W. Hörz, Cell **42,** 281–290 (1985).
[14] T. Igo-Kemenes, A. Omori, and H. G. Zachau, Nucleic Acids Res. **8,** 5377–5390 (1980).
[15] H. Böck, S. Adler, X.-Y. Zhang, H. Fritton, and T. Igo-Kemenes, J. Mol. Biol. **176,** 131–154 (1984).
[16] F. Strauss and A. Varshavsky, Cell **37,** 889–901 (1984).
[17] M. J. Solomon, F. Strauss, and A. Varshavsky, Proc. Natl. Acad. Sci. U.S.A. **83,** 1276–1280 (1986).
[18] T. Hsieh and D. Brutlag, Proc. Natl. Acad. Sci. U.S.A. **76,** 726–730 (1979).
[19] I. Levinger and A. Varshavsky, Cell **28,** 375–385 (1982).
[20] I. Levinger and A. Varshavsky, Proc. Natl. Acad. Sci. U.S.A. **79,** 7152–7156 (1982).
[21] A. Collick and A. J. Jeffreys, Nucleic Acids Res. **18,** 625–629 (1990).
[22] W. P. Wahls, G. Swenson, and P. D. Moore, Nucleic Acids Res. **19,** 3269–3274 (1991).

[23] H. Garreau and J. G. Williams, Nucleic Acids Res. **11,** 8473–8484 (1983).
[24] J. Avilla, E. M. de Garcini, F. Wandosell, A. Villasante, J. M. Sogo, and N. Villanueva, EMBO J. **2,** 1223–1234 (1983).
[25] J. D. Lewis, R. R. Meehan, W. J. Henzel, I. Maurer-Fogy, P. Jeppesen, F. Klein, and A. Bird, Cell **69,** 905–914 (1992).
[26] X.-Y. Zhang, K. C. Ehrlich, P. C. Supakar, and M. Ehrlich, Mol. Cell Biol. **9,** 1351–1356 (1989).
[27] J. Timmis and J. Ingle, Biochem. Genet. **15,** 1159–1173 (1977).
[28] V. Hemleben, B. Leweke, A. Roth, and I. Stadler, Nucleic Acids Res. **10,** 631–644 (1982).
[29] C. Ramachandran, W. A. Brandenburg, and A. P. M. den Nijs, Plant Syst. Evol. **151,** 31–41 (1985).
[30] M. Ganal, I. Riede, and V. Hemleben, J. Mol. Evol. **23,** 23–30 (1986).
[31] M. Ganal and V. Hemleben, Theor. Appl. Genet. **75,** 357–361 (1988).
[32] C. Yanisch-Perron, J. Viera, and J. Messing, Gene **33,** 103–119 (1985).
[33] F. Sanger, S. Nicklen, and A. R. Coulson, Proc. Natl. Acad. Sci. U.S.A. **74,** 5463–5467 (1977).
[34] J. Pustell and F. C. Kafatos, Nucleic Acids Res. **12,** 643–655 (1984).
[35] M. M. Bradford, Anal. Biochem. **72,** 248–254 (1976).
[36] U. Zentgraf and V. Hemleben, Nucleic Acids Res. **20,** 3685–3691 (1992).
[37] W. Hörz and W. Altenburger, Nucleic Acids Res. **12,** 2643–2659 (1981).
[38] C. Dingwall, G. P. Lomonossof, and R. A. Laskey, Nucleic Acids Res. **9,** 2659–2133 (1981).
[39] A. A. Travers, Annu. Rev. Biochem. **58,** 427–452 (1989).
[40] T. Beridze, N. Tsiredkidze, and M. A. Roytberg, Biochimie **74,** 187–194 (1992).
[41] S. Spiker, Annu. Rev. Plant Physiol. **36,** 235–253 (1985).